Characterization of Lipopeptides Biosurfactants Produced by a Newly Isolated Strain *Bacillus Subtilis* ZNI5: Potential Environmental Application

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Abstract

Strain ZNI5, isolated from a hydrocarbon contaminated soil and identified as *Bacillus subtilis* after 16s rDNA sequence, grew and produced lipopeptides biosurfactants when cultured on glucose based media. After purification by anionic exchange chromatography and identification Reverse Phase High Performance Liquid Chromatography-Mass Spectrometry, the biosurfactant produced by ZNI5 were determined to be cyclic lipopeptides homologues. Four families of lipopeptides were identified by HPLC-MS analysis. They belongs to surfactin isoforms with molecular weights of 979, 993, 1007, 1021 and 1035 Da; iturin isoforms with molecular weights of 1028, 1042 and 1056 Da; Licheniformin with molecular weight of 1410 and newly identified isoforms named Inesfactin with molecular weights of 973 and 987 Da. Functional properties of the ZNI5 biosurfactant were studied. It was characterized as a powerful surface-active agent that decreases the surface tension of water from 72 mN/m to about 32 mN/m with a CMC value of 350 mg/L more efficient than chemical surfactants (Triton X100; CTAB and SDS). It has the capacity to disperse oil to about 80 mm at a concentration of 800 mg/L showing close efficiencies to the listed chemical surfactants. In addition, by studying the surface tension decrease capacity and the oil displacement activity, ZNI5 lipopeptide biosurfactant showed great thermal, pH and salts activity and stability enabling its use in the bioremediation fields and for diverse industrial applications.

Introduction

Biosurfactants surface-active substances synthesized by living cells (bacteria, yeast and fungi) They are amphiphilic compounds with a hydrophilic (amino-acid or peptides; di- or polysaccharides; anions or cations) and hydrophobic moieties (saturated or unsaturated fatty acid) (Mnif &Ghribi 2015a). Like chemical surfactants, biosurfactants are well known by their ability to decrease the surface and interfacial tension at the surface and interface respectively (Mnif &Ghribi 2015a). Owing their large diversity, environmentally friendly nature, non-toxicity, large biodegradability and possibility of large-scale production, a great interest was spotted onto biosurfactants in recent years. In addition to these properties, their numerous functional and biological activities and ability to function under extreme conditions of temperature, pH and salinity permit their application in various fields including food, agriculture, biomedical and environment (Mnif &Ghribi 2015a, Mnif &Ghribi 2015b, Mnif &Ghribi 2015c). They appear as best replacements of chemical surfactants and scientists focus on the research of new-biosurfactant producing microorganisms. When grown on immiscible or miscible substrates, these microorganisms synthesize a wide range of chemicals with surface active compounds, with diverse nature (Mnif &Ghribi 2015a). Owing their biochemical nature, biosurfactants are classified onto six groups including glycolipids, lipopeptides, phospholipids, lipopolysaccharides, neutral lipids and polymeric surfactants (Mnif &Ghribi 2015a). Glycolipids and lipopeptides consisting of a fatty acid chain linked to a carbohydrate and peptidic moieties respectively are among the most recognized biosurfactants with a high structural versatility and various functional activities (Mnif &Ghribi 2015a).

Lipopeptides, are characterized by diverse functional properties (emulsification/de-emulsification, dispersing, foaming, viscosity reducers, solubilizing and mobilizing agents, pore forming capacity) and endowed by different biological activities (antimicrobial; hemolytic; antiviral; antioxidant …) permitting their use in many domains (Mnif &Ghribi 2015c, Mnif &Ghribi 2015d). Especially, having the ability to emulsify, solubilize and mobilize hydrocarbon contaminants, lipopeptides increases their availability for microbial degradation (Mnif &Ghribi 2015c), (Mnif et al. 2014, Mnif et al. 2013b, Mnif et al. 2015a, Mnif et al. 2017a, Mnif et al. 2013a). They are very advantageous over synthetic emulsifiers for the enhancement of the bioremediation of hydrocarbon contaminated sites (Mnif &Ghribi 2015c, Mnif &Ghribi 2015d). Also, lipopeptide biosurfactants stimulates dyes bio-decolorization (Mnif et al. 2015b, 2015c, Mnif et al. 2016a). Moreover, they can be used in enhanced oil recovery and may be considered for other potential applications in environmental protection (Mnif &Ghribi 2015c). In addition, they can be applied in herbicides and pesticides formulations, detergents, healthcare and cosmetics, pulp and paper, coal, textiles, ceramic processing and food industries (Bouassida et al. 2018, Mnif &Ghribi 2015c).

Oil spills in the oceans causes great damage to local animal, plant life and disturb all the ecological equilibrium (Song et al. 2013). Due to increasing exploitation, production, transportation and storage, oil spread over a large area on sea surface causing great damage. The application of chemical dispersants, defined as a material that reduces the cohesive attraction between similar particles, is an efficient mean that help in the mechanic restoration of oil spills (Mnif et al. 2017b). Dispersant agents prevent insoluble particles to form aggregations with each other that keep insoluble particles in suspension and oil slicks are broken up. When they are sprayed onto oil slicks, they accelerate the dispersion of oil from the sea surface enhancing their removal (Guodong et al. 2015). During oil dispersion, the hydrophilic part of the surfactant turns towards the hydrophilic phase “the sea water” while the hydrophobic tail of the molecule turns towards the oil phase, leading to the formation of small oil droplets that are stabilized by the dispersant (Guodong et al. 2015, Pi et al. 2015). In addition to oil dispersion, dispersants agents have application in oil chemistry field as they have great role in desorption of hydrophobic molecules from rock surfaces enhancing their mobility and recovery. However, chemical dispersants have great hazardous effects to marine and soil ecosystems and cause great toxicities to living organisms (Kleindienst et al. 2015, Popovech 2017). Also, they can suppress the activity of oil degrading micro-organisms when applied to the oil-contaminated seawater to disperse surface slicks into smaller droplets that are presumed to be more bio-available to microorganisms (Kleindienst et al. 2015). So, an urgent need for natural and environment-friendly oil spill dispersants was developed (Guodong et al. 2015). Owing their great surface activity, biodegradability and non-toxicity, biosurfactants can be an eco-friendly alternative to chemical dispersants. Many previous reports described the use of biosurfactants as oil dispersants promoting therefore hydrocarbon decontamination and environment cleaning (Da Silva et al. 2017, Freitas et al. 2016, Rongsayamanont et al. 2017, Song et al. 2013).

Here, we report the screening and the identification of a newly biosurfactant producing bacterium. After that, biosurfactant purification and identification was studied by an acid precipitation followed by anionic exchange chromatography and High Performance Liquid Chromatography coupled to a mass Spectrometry. Aiming its potential use in bioremediation, we studied the surface tension decreasing

capacity and the oil dispersant activity the produced biosurfactant along with the effect of different physic-chemical factors on these activities.

Materials And Methods

Materials

Motor oil used in this study was obtained from a local Mechanic's station (Sfax, Tunisia). An anionic chemical surfactant Sodium Dodecyl Sulfate (SDS), a cationic chemical surfactant Cetyl Bromure Trimethyl Ammonium (CTAB) and two non-ionic surfactants Tween 80 and Triton X100 were purchased from Sigma. They were dissolved in distilled water for the measurement of their surface tension decrease and oil dispersion activity.

Bacterial isolation

Biosurfactant-producing strains were screened from contaminated soil near the Tunisian Chemical Group of Gabes (Gabes, Tunisia). The procedure of isolation was done as described by (Ghribi et al. 2012). A soil suspension was prepared by suspending 1 g of soil sample in 9 mL of saline water. From this, serial dilutions (up to 10^{-8}) of soil samples were carried in 0.9 % sterile saline. A 0.1 mL from each diluted samples was plated onto the surface of Lauria Bertani agar medium. The plates were then incubated at 37°C for 24 hours. Pure cultures with different morphological properties were obtained after repetitive streaking on Lauria–Bertani (LB) agar medium.

Screening of biosurfactant producing strain

Firstly, blood haemolysis technique was applied as a preliminary screening method of biosurfactants producers (Ghribi et al. 2012). Pure cultures of the isolated strains were streaked on blood agar. After incubation at 37 ℃ for 24 hours, biosurfactants production appeared as clearing zone around the colonies. Secondly, bacteria having hemolytic activities were screened for surface tension decrease on liquid media (Mnif &Ghribi 2015a). For this, they were cultivated on glucose containing media for two days. Supernatants were separated from the culture media by centrifugation and they were subjected to surface tension measurement. It was measured according to the Wilhelmy method using a tensiometer"TD2 LAUDA".

Identification of the biosurfactant producing bacteria by the 16 rDNA gene sequencing

Bacterial isolate which displayed high biosurfactant production was selected in order to be identified. PCR amplification of 16S rDNA gene with the universal primers Forward Fd1 (5'AGTTTGATCCTGGCTCAG3') and Reverse Rd1 (5'AAGGAGGTGATCCAGCC3') was carried out and was directly sequenced (Mnif et al. 2016a, Mnif et al. 2015a). The 25 μL of PCR mixture contained: 0.2 mM of deoxy nucleoside triphosphate, 1.32 μM concentration each primer, 0.5U of DNA polymerase, 5 μL of 5X buffer and 1 μL (5 ng) of total DNA template. The PCR program was 94 ℃ for 3 min followed by 35 cycles

consisting of denaturation at 94°C for 45 second, annealing at 59 ℃ for 1 min and extension at 72 ℃ for 2 min. Following amplification, the PCR product was purified (Promega Gel Extraction Kit, Biogène, Tunisia) and sequenced with ABI PRISMTM 3100 Genetic Analyzer (Applied Biosystems, USA). The resulting sequences were aligned and compared with sequences in the Gene Bank database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) using the nucleotide–nucleotide blast (BLASTn) network service (Mnif et al. 2015a). Phylogenetic tree was draw using the software MEGA version 10 by the neighbor joining method.

Culture media and cultivation conditions

Bacterial strain was streaked on a LB agar medium and incubated at 37 ℃ (Ghribi et al. 2012). After 24 h, one loop of cells was dispensed in 50 ml LB medium prepared into a 250 mL shake flask containing 50 mL LB liquid medium: 10.0 g/L peptone, 5.0 g/L yeast extract, 5.0 g/L NaCl. Inoculum culture was cultivated at 37°C with shaking at 180 rpm for 18 h. For biosurfactant production, four percentage (v/v) inoculums was transferred into a 250 mL shake flasks containing 50 mL of a modified Glucose based medium which contains: 20.0 g/L glucose, 5.0 g/L yeast extract, 1.5 g/L KH_2PO_4, 0.1 g/L KCL, 0.5 g/L $MgSO_4$, 0.008 g/L $FeSO_4$, 0.05 g/L $CaCl_2$, 0.1g/L KCl and traces elements (Cu, Mn, Zn, Br) (Landy et al. 1948) with little modifications). The initial pH values of the media were adjusted to 7.0. Cultures medium were incubated for 48 hours under shaking at 150 rpm.

Biosurfactant recovery

For test motor oil dispersion study and surface tension measurement, lipopeptide biosurfactants were partially purified during three consecutive cycles of acid precipitation–dissolution as described by (Mnif et al. 2013b, Mnif et al. 2013a). The resultant biosurfactants pellets collected by centrifugation at (8000 rpm/4°C/20 min), washed twice with acid distilled water (pH = 2) to eliminate any impurities, dissolved in distilled water and the pH was adjusted to 8.0 by 1 N NaOH to dissolve the most lipopeptide compounds. After that, the final dissolved extract was lyophilized (Mnif et al. 2013b, Mnif et al. 2013a). This serves as crude lipopeptide preparation to perform the surface tension measurement test and dispersion activity study.

Biosurfactant purification by anionic exchange chromatography and RP-HPLC

The crude extract was applied to a low-pressure chromatography column filled with Bio-Rex 70 (Pharmacia, Uppsala, Sweden) equilibrated in 50 mM Tris-HCl, pH 8. Preliminary studies demonstrated that biosurfactant compound was retained on the Bio-Rex 70. Fractions of 5 ml were eluted with a gradient of NaCl ranging from 0.1 to 0.8 M NaCl in buffer (50 mM, pH 8) at a flow rate of 1.0 ml/ml. The eluant was monitored at 280 nm, and fractions were collected every 5 min (Bechard et al. 1998). The different fractions were subjected to surface tension and dispersion activities measurement. Active fractions were pooled and concentrated by acid precipitation (at pH = 2) followed by centrifugation. The resulted pellets were dissolved in ultrapure water and the pH was adjusted to 8.0 using 1 N NaOH and surface activities were measured again.

Active fractions were purified and identified by RP-HPLC coupled with mass detector. The HPLC system (Shimadzu, Japan) consisted of two pumps, a column oven, a sample injector and a mass detector (Shimadzu, Japan). The analytical HPLC column was a C18 reversed phase column, 5 µm, 250 × 4.6 mm (Elite, China). The HPLC-MS system (Agilent 1100 Liquid Chromatography-LCQ Deca XP Ion Trap Mass) was used to analyze the products. A mobile phase was prepared as a mixture of Methanol (A) and redistilled water (B) in different proportion. The gradient of 80 % (A) and 20 % (B) was maintained in the first 2 min, and then increased from 80 to 100 % (A) for the next 18 min. After 20 min, it's maintained at 100 %. A constant flow-rate and temperature of 1.0 mL/min and 30°C respectively were maintained during the HPLC analysis.

Functional characterization of crude lipopeptide preparation

Surface tension measurement and determination of CMC

A model Tensiometer Sigma 700 was used to measure the surface tension of the crude lipopeptide preparation according to the Du Noüy Ring method. Measurements were carried out at room temperature exception special indication. Surface tension was determined in function of increasing concentration of biosurfactant concentration prepared in distilled water. When an abrupt decrease of the surface tension was reached, the Critical Micelle Concentration (CMC) was attained. It corresponds to the concentration at which biosurfactants associates into micelles.

Study of oil dispersion activity

Dispersion capacity of motor oil was studied by oil displacement test. Firstly, 40 mL of distilled water were added to a Petri dish with a diameter of 15 cm. After that, 20 µL of motor oil was dropped onto the surface of the water in the center of the petri dish, followed by the addition of 10 µL of the crude biosurfactant. The oil dispersion capacity corresponds to the diameter of the clear zone that appears at the surface of water (Rodrigues et al. 2006).

Effects of physic-chemical factors on surface tension reduction and oil dispersion activities and stabilities

To study the effect of different physicochemical factors on ZNI5 biosurfactant, oil dispersion and surface tension reduction activities were measured at different pH, Temperature and NaCl concentration values. Experiments were performed as described above for test displacement assay with 0.1 % biosurfactant solution. For the surface activity determination, experiments were conducted at the Critical Micelle Concentration. The effect of pH buffer on the two activities was evaluated at different pH values ranging from 2.0 to 10.0 using glycine–HCl buffer (pH 2.0–3.0), acetate buffer (pH 4.0–5.0), phosphate buffer (pH 6.0–8.0) and glycine–NaOH buffer (pH 9.0–10.0) (all at a final concentration of 20 mM) (Mnif et al. 2013b, Mnif et al. 2013a). Results were expressed as residual activity towards dispersion activity at neutral pH. For the determination of salt effect, different quantities of NaCl were dissolved in buffer solutions to adjust the salt concentration of test samples to 0, 0.25, 0.5, 1, 2.5, 5, 7.5 and 10 % (weight/volume). Results were expressed as residual activity towards activities without salt addition

(Ghribi et al. 2012). Also, we evaluated the activities at different temperature ranging from 4°C to 80°C. Residual activity was determined towards the activity measured at ambient temperature.

Similarly, the stability of the biosurfactant was evaluated after pre-incubation at different pH buffers, different salt concentration and different temperature by measuring the oil dispersion and the surface tension reduction activities. All results were expressed by relative activity towards biosurfactants activities without incubation.

Results And Discussion

Strain selection and identification of the newly producing biosurfactant

Life in the polluted environment is limited by the extreme conditions, and only pollution-adapted microorganisms and certain animals and plants survive. The region of Gabes is polluted by the chemical industry, although the climate is arid and the temperature was high, where the sample analyzed here was collected. Its isolation as a polluted environment and extreme climatic conditions may have allowed the development in of bacteria with unique metabolic profiles. Nowadays, biosurfactants are very used in different domain especially in pharmaceutical, cosmetic, agro-food industry and environmental field. Owing to their lower toxicity, higher biodegradability and efficient activities at extreme pH and temperature, they became great replacers of chemical surfactants. In this aim, scientists focus on the isolation of new biosurfactant producing strain. For that purpose, seventy strains isolated from a contaminated soil of the region of Gabes were screened for their ability to produce biosurfactants by using blood hemolysis assay (Mnif &Ghribi 2015a). In fact, hemolytic activity can be applied as a preliminary screening method predicting the possibility of biosurfactant production and must be complemented by the measure of surface tension and oil dispersing activity. Biosurfactant activity can be detected by the ability to decrease the surface tension of water and provoke a clearing zone on an oil surface. Many previous studies reported the use of the oil spreading test to detect biosurfactant production (Elazzazy et al. 2015, Jemil et al. 2016, Marajan et al. 2015). Among the seventy isolated strains, five strains having the highest hemolytic halos were selected and subjected to surface tension and oil displacement activities measurements. Among the five strains screened, one unique strain showed the highest decrease of surface tension of the medium to about 21 mN/N with a higher oil dispersing capacity. It was subjected to a molecular identification by amplification of the 16 S rDNA.

After PCR amplification, sequencing and phylogenetic analysis, strain ZNI5 was identified as *Bacillus subtilis* — a saprophytic bacterium widespread in nature. The amplified sequence was compared with those in the Gen-Bank nucleotide database by a BLAST search analysis and showed high identity scores with members of the *Bacillus subtilis* species. A phylogenetic tree was drawn using Mega 10 program and showed the nearest neighbor joining to *Bacillus subtilis* strains. The 16S rDNA gene sequence was deposited in the Gen-Bank database under accession number MW091416.

The number of newly described species of *Bacillus subtilis* described to produce biosurfactants has grown considerably in the last 20 years. They were selected as safe micro-organisms and higher producers of secondary metabolites having interesting surface active compounds.

Purification and Identification of ZNI5 lipopeptide biosurfactants

As described in Materials and Methods section, the crude lipopeptide extract prepared after three cycle of acid-precipitation followed by a lyophilisation. After that, it was purified by an anionic exchange chromatography filled with BioRex 70 resine. Two active fractions were pooled separately. The surface activity of the fractions was verified by measuring the surface tension reduction and the oil dispersion activity. Being active, these two fractions were passed through C18 column chromatography coupled is a mass detector to determine the molecular weight of the constituting fractions. Obtained results showed four clusters of peaks, the first at m/z values between 973 and 987 Da (Family A), the second at m/z values between 979 and 1035 Da (Family B), the third at m/z values between 1028 and 1056 Da (Family C) and the fourth at m/z value of 1410 Da (Family D) (Table 1). Family A corresponds to two newly identified lipopeptides named Inesfactin as described in our previous work reporting the identification of lipopeptides isorforms derived from *Bacillus subtilis* SPB1. Family B and C correspond to the largely identified isoforms of surfactin and iturin compounds. Surfactin isoforms with molecular weights of 979, 993, 1007, 1021, and 1035 Da corresponding to C11; C12; C13; C14 and C15 Surfactin were identified as largely described in previous studies (Ben Ayed et al. 2014, Chen et al. 2020, Hentati et al. 2019, Jemil et al. 2019, Kecskeméti et al. 2018, Mnif et al. 2016b, Yang et al. 2015). Iturin isoforms with molecular weights of 1028, 1042, and 1056 Da corresponding to C13; C14 and C15 Iturin were identified as largely reported in recent studies (Gong et al. 2015, Wang et al. 2020, Yang et al. 2015, Zhao et al. 2016, Zhou et al. 2020). Family D constituted with a unique compound having a molecular weight of 1410 Da may belong to Licheniformin family as published by (Biria et al. 2010).

Table 1
Different lipopeptides identified by High Performance Liquid Chromatography coupled with a mass detector

Family	Mass peak (m/z)	Lipopeptide nature	References
Family A	974 973	Inesfactin [M + H] $^{+}$ Inesfactin	(Mnif et al. 2016b)
	988 987	Inesfactin [M + H] $^{+}$ Inesfactin	
Family B	980 979	C11-Surfactin [M + H] $^{+}$ C11-Surfactin	(Ben Ayed et al. 2014, Chen et al. 2020, Hentati et al. 2019, Jemil et al. 2019, Kecskeméti et al. 2018, Mnif et al. 2016b, Yang et al. 2015)
	994 993	C12-Surfactin [M + H] $^{+}$ C12 - Surfactin	
	1008 1007	C13-Surfactin [M + H] $^{+}$ C13-Surfactin	
	1022 1021	C14-Surfactin [M + H] $^{+}$ C14-Surfactin	
	1036 1035	C15-Surfactin [M + H] $^{+}$ C15-Surfactin	
Family C	1029 1028	C13-Iturin [M + H] $^{+}$ C13-Iturin	(Gong et al. 2015, Wang et al. 2020, Yang et al. 2015, Zhao et al. 2016, Zhou et al. 2020)
	1043 1042	C14-Iturin [M + H] $^{+}$ C14-Iturin	

Family	Mass peak (m/z)	Lipopeptide nature	References
	1057 1056	C15-Iturin [M + H] $^{+}$ C15-Iturin	
Family D	1411 1410	Licheniformin [M + H] $^{+}$ Licheniformin	(Biria et al. 2010)

Structurally, lipopeptides are a combination of a hydrophilic head and a hydrophobic tail. They consist of a fatty acid chain linked to a short linear chains or cyclic structures of amino acids via ester or amide bonds or both (Mnif &Ghribi 2015c). A lactone bridge between the β-hydroxyl function of the acid and the carboxyl-terminal function of the peptide confers a cyclic structure to this molecule (Mnif &Ghribi 2015c). Owing to the nature of the fatty acid chain, as well as the type, the number and the configuration of the amino acids in the peptide chain, lipopeptides are classified into different families (Mnif &Ghribi 2015c). Cyclic lipopeptides belonging to surfactin, fengycin and iturin families, identified in our present work, are among the three major classes of lipopeptide biosurfactant isoforms produced by *Bacillus* strains. The different families differ by the composition of the amino acid sequence and the different isomers of each family are characterized by a constant peptide moiety with difference in the length of the fatty acid chain for the lipid moiety (Mnif &Ghribi 2015c).

For surfactin, the peptide part is a cyclic lipoheptapeptide, containing seven residues of D- and L-amino acids and one residue of a β- hydroxy fatty acid. Regarding the length of the lipid moiety, surfactin can have 6 isoforms or 9 isoforms (Mnif &Ghribi 2015c). However, the diversity of the peptide moiety permits to distinguish surfactin, lichenysin, esperin and pumilacidin among the surfactin family that have difference on the seventh amino-acid (Mnif &Ghribi 2015c). Surfactin is well characterized by diverse functional properties and biological activities. It is able to reduce surface tension of water from 72 to 27 mN/m at 0.005 % with potent antibacterial, antiviral, antymicoplasma, antitumoral and anticoagulant as well as its inhibitors roles of enzymes (Mnif &Ghribi 2015c).

For iturin, they share a common sequence of a β-hydroxy fatty acid-Asx-Tyr-Asx) and show variation at the other four positions of the peptide moiety (Mnif &Ghribi 2015c). According to the nature of the rest of the peptide ring, Iturin are classified into Iturin A, C, D and E, Bacillomycin D, F and L, Bacillopeptin and Mycosubtilin (Mnif &Ghribi 2015c). Having the ability to permeabilize membranes, iturin are qualified as a special class of pore-forming lipopeptide permitting their use as anti-microbial agents. They have great antifungal activity against a wide variety of pathogenic yeasts and fungi against a restricted antibacterial activities to some bacteria species (Mnif &Ghribi 2015c).

Licheniformin have been characterized by (Biria et al. 2010). It presents an unique structure with amino acid sequence of Gly, Ala, Val, Asp, Ser, Gly, Tyr and a lactone linkage between the carboxyl group of Aspargine and hydroxyl group of Tyrosine residue and an fatty acid moiety constituted of 12 carbon attached to N-terminal amino acid residue through an amide bond (Biria et al. 2010).

Study of the surface tension decrease and oil dispersing capacity of ZNI5 lipopeptide biosurfactant

Surface tension measurement and determination of the Critical Micelle Concentration (CMC); Comparison with chemical surfactants

Generally, surface tension corresponds to the free energy per unit area associated with a surface or an interface that can be (Mnif &Ghribi 2015a). It can be easily measured with a tension-meter. Biosurfactants are surface active molecules that have the ability to accumulate onto the surfaces liquid or the interfaces between two immiscible liquids decreasing the surface or the interfacial tension respectively (Mnif &Ghribi 2015a). A gradual decrease of the surface tension is observed as function of the increase of the surfactant concentration up to a critical level known as the Critical Micelle Concentration (CMC) (Mnif &Ghribi 2015a, Pacwa-Płociniczak et al. 2011). Above this value, surfactant molecules associate readily to form supra-molecular structures like micelles, bilayers and vesicle (Mnif &Ghribi 2015a). The profile of surface tension as a function of biosurfactants concentration is illustrated in Fig. 1. *B. subtilis* ZNI5 lipopeptide biosurfactant decreases the surface tension of water from 72 mN/m to about 32 mN/m called the γCMC and achieves the CMC at 350 mg/L. To know, the values of surface tension, interfacial tension and CMC value characterize each biosurfactant and measure its efficiency (Mnif &Ghribi 2015a). By analyzing the literature, the CMC value of our biosurfactants and its ability to reduce the surface tension vary between several studies. It shows better efficiency in comparison to numerous bacterial derived lipopeptides as described in Table 2. However, it’s less efficient (Diniz Rufino et al. 2014). These varieties can due to the different lipopeptides produced, the substrate of production and the producing strains.

Table 2
Comparative study among different bacterial derived lipopeptides; lipopeptides properties and stabilities

Strains	Substrate for BS production	Biosurfactant properties	Biosurfactant stability	Study
B. subtilis ZNI5	Glucose	CMC = mg/L ST = mN/m		Our study
B. natto TK-1	Sucrose	CMC = 512 mg/L ST = 30.1 mN/m antimicrobial and anti-adhesive activities	-	(Cao et al. 2009)
B. subtilis ATCC 21332		CMC = 250 mg/l ST = 27.9 mN/m		(Cao et al. 2009)
B. cereus	Frying oil	CMC = 500 mg/L ST = 27 mN/m EI = 90% (Motor oil) Enhanced the degradation of motor oil up to 96% Displacement activity	Stability at pH (2–10), salinity (2–10%), and temperature (5–120°C) Stable after heating of 90°C for 120 min.	(Durval et al. 2019)
B. coagulans	-	CMC = 17 mg/L ST = 29 mN/m	-	(Diniz Rufino et al. 2014)
B. mojavensis I4	Glucose	CMC = 100 mg/L ST = 29 mN/m EI24 = 62%	Temperatures (4–120°C) pH (4–10) Salinity (2–12% of NaCl)	(Ghazala et al. 2019, Ghazala et al. 2017)

Strains	Substrate for BS production	Biosurfactant properties	Biosurfactant stability	Study
B. methylotrophicus DCS1	Diesel oil	CMC = 100 mg/L ST = 31 mN/m E24 = 62%	pH, temperature and salinity stabilities	(Jemil et al. 2016)
B. subtilis SPB1	Glucose	CMC = 17 mg/L ST = 29 mN/m Emulsifying, solubilizing and mobilizing activities	pH, temperature and salinity	(Mnif et al. 2013b)
Corynebacterium aquaticum	Fish residue	ST = 27.8 mN/m E24 = 87.6%	temperatures of 20 to 121 °C, in saline concentrations of 1 to 7%, and at pH close to neutrality	(Martins et al. 2018)
B. licheniformis DS1	Crude oil	EI24 = 65.19% CMC = 157.5 mg/L	pH 4–10, high temperatures up to 120°C, and with an NaCl concentration up to 10% (w/v)	(Purwasena et al. 2019)
B. subtilis K1	Glucose	CMC = 20.5 µg/mL Emulsification activity	100°C for 2 h, over a pH range of 6–12 h and over an NaCl concentration up to 10 % (w/v).	(Mnif et al. 2013b, Pathak &Keharia 2014)
B. nealsonii S2MT	Glycerol	34.15 ± 0.6 mN/m EI = 55 ± 0.3%) for kerosene oil CMC= 40 mg/L Enhanced the remediation of heavy engine-oil contaminated soil	highly stable at high temperature, at neutral and alkaline pH and moderate salt concentration	(Phulpoto et al. 2020)

Strains	Substrate for BS production	Biosurfactant properties	Biosurfactant stability	Study
B. subtilis N3-1P	FL and Fish waste-based peptone	ST = 27.9 mN/m and 27.8 mN/m 0.18 g/L and 0.3 g/L	The surface tensions remained in a narrow window of 35.2–27.3 mN/m from 0°C to 100°C; stable at a pH range from 4 to 12.	(Zhu et al. 2020)
Bacillus sp.MSI 54	Lactose	ST = 30.0 mN/m CMC = 10 mg/L	The emulsification index (81%) was retained even at pH levels of 4.0–9.0 and salt concentrations of 2–12% and at temperature from 4 to 121°C	(Ravindran et al. 2020)

As it is known, higher toxicity of chemical surfactants to the environment and living organisms limits their application in environmental field (Lechuga et al. 2016, Lémery et al. 2015, Rebello et al. 2014). Therefore, biosurfactant became a best alternative. In this aim, ZNI5 biosurfactant efficiency was compared with those of chemical surfactants; Triton X100 (γCMC = 32 mN/m, CMC = 200 mg/L); CTAB (γCMC = 35 mN/m, CMC = 31 mg/L) and SDS (γCMC = 35 mN/m, CMC = 794 mg/L). Obtained results showed higher efficiencies of ZNI5 lipopeptide towards the three studied chemical surfactants. They are similar to those published by (Pornsunthorntawee et al. 2008) showing a better efficiency of a *P. aeruginosa* derived surfactants than synthetic surfactants. Generally, biosurfactants are characterized by smaller critical micelle concentration (CMC) than the synthetic surfactants with low γCMC values making them better and more efficient (Pornsunthorntawee et al. 2008). Similarly, our previous works demonstrated the efficiency of *B. subtilis* SPB1 towards chemical surfactants in the bioremediation of polluted environment by dyes and hydrocarbons (Mnif et al. 2015b, 2015c, Mnif et al. 2016a, Mnif et al. 2015a, Mnif et al. 2017a). To conclude, the higher efficiency of bacterial derived surfactants in addition to their non-toxicity to living organisms and their biodegradability permit their use as best alternative to chemical surface-active compounds for different applications (Mnif &Ghribi 2015c).

Study of oil displacement ability

In addition to the ability to reduce the surface tension, biosurfactants have the capacity to displace oils evaluated by the diameter of clearing zone on the surface of water (Cao et al. 2009). To define, a dispersants is a material that reduces the cohesive attraction between similar particles. With this, it keeps insoluble particles in suspension by preventing insoluble particles to form aggregations with each other (Cao et al. 2009). Generally, lipopeptide biosurfactants have the ability to disperse oil layer and provoke a clearing zone on an oil surface (De Almeida et al. 2016, Liu et al. 2015, Silva et al. 2014). Preliminary study showed a better displacement capacity of motor oil. As described in Fig. 2, the diameter of oil displacement increase linearly as function of ZNI5 biosurfactant concentration to achieve constant value at an optimal concentration. Obtained results showed an interesting motor oil displacement of about 60 mm for ZNI5 biosurfactant concentration ranging from 300 to 600 mg/L and to reach 80 mm for

biosurfactant concentration equal or superior to 800 mg/L. To ensure the motor oil dispersing activity of ZNI5 lipopeptide preparation, we added two photos visualizing the motor oil before dispersion and the motor oil after dispersion with a very large diameter of dispersion. Figure 3 visualize the motor oil before dispersion and after dispersion. Excellent motor oil dispersion is ensured by a very large diameter of dispersion by the crude ZNI5 lipopeptide preparation. The dispersion appeared instantly after the spot of the lipopeptide on the center of the motor oil drop. Numerous previous studies reported the efficient use of bacterial derived lipopeptide as dispersant agents (Feng et al. 2019, Freitas et al. 2016, Ghazala et al. 2019) (Jemil et al. 2016, Rongsayamanont et al. 2017). A comparative study of ZNI5 lipopeptide towards chemical surfactants was studied. Diameters of oil displacement were of about 92 mm ; 88 mm and 85 mm for Triton X100 at 1000 mg/L ; CTAB at 1000 mg/L and SDS at 85 mg/L respectively. Therefore, chemical surfactants as well as ZNI5 biosurfactant show close efficiencies. Results are similar to those published by (Silva et al. 2014) showing the similar dispersing power of certain oils by a derived *Cunninghamella echinulata* polymeric surfactant and Triton X100. Nevertheless, previous studies reported the effectiveness of certain biosurfactants in the dispersion of hydrocarbons towards chemical surfactants like SDS and Tween 80 (Chandran &Das 2010), Tween 20; Tween 80; Triton X100 and SDS (Qiao &Shao 2010) and SDS (Feng et al. 2019). However, ZNI5 lipopeptide biosurfactant can be a best alternative to chemical surfactants as surfactants are often more toxic than the oil that is being dispersed, and the combination of the oil and the surfactant can be more toxic than either alone (Negin et al. 2017). Therefore, as described by (Kleindienst et al. 2015), the application of chemical surfactants as oil dispersants can inhibits hydrocarbon degrading bacteria. Also, it is worthy to note that surfactants are some of the most expensive chemicals used during Enhanced Oil Recovery (Negin et al. 2017). So, ZNI5 biosurfactant offer a big potential of application as low cost, healthier and more ecological alternative oil dispersant.

The efficiency of motor oil displacement offers a high potential of application of ZNI5 biosurfactant in bioremediation. Indeed, the massive use of petroleum products contributes enormously to the water pollution causing a major environmental risk of human health. The strong hydrophobic character of oils and hydrocarbons and their non-solubility limits their microbial biodegradation and elimination. Their bio-dispersal on the surface of waters facilitates their mechanic and microbial elimination (Freitas et al. 2016, Rongsayamanont et al. 2017, Silva et al. 2014). Moreover, dispersants can lead desorption of hydrophobic molecules from rock surfaces enhancing mobility and recovery in oil field chemistry (Da Silva et al. 2017).

Effects of physic-chemical factors on oil dispersion and surface tension reduction activities and stabilities

Study of biosurfactant activity at different temperature, pH and salinities

In order to be applied in industrial process, the surface activity of ZNI5 biosurfactant was assayed at different pH, temperature and salt concentration values. Activities were evaluated by the determination of

surface tension reduction using 350 mg/L biosurfactant and oil dispersion capacity using 1000 mg/L.

The thermal activity study of ZNI5 biosurfactant activity demonstrated a maximal surface tension reduction and oil dispersion activity at 20 and 30°C. Beyond these temperatures, we observed a slight decrease (Fig. 4). For the surface tension reduction, we observe a gradual decrease of the surface activity of ZNI5 lipopeptide with the increase of temperature to reach 45.6 % of its activity at 100°C. However, the results obtained show that ZNI5 lipopeptide has the ability to disperse the engine oil even at very high temperature. In fact, it keeps about 77 % of its relative activity at 100°C. Nevertheless, this biosurfactant shows interesting thermal activity as it retains more than 50% of its activity at temperatures below or equal to 80°C. Results are similar to that obtained by (Mnif et al. 2013b) and (Feng et al. 2019).

Regarding the pH activity, obtained results showed that ZNI5 lipopeptide is active in a wide pH range (Fig. 5). In fact, it retains between 90 and 100 % of its surface tension reducing power and dispersant activity at pH 5 to 10. However, a decrease of its surface activity is observed at highly acidic pH ranging from 2 to 4. This can be attributed to the lipopeptide nature of biosurfactants as they precipitate at lower pH values below 5 and are totally soluble at neutral pH and slightly alkaline pH (Takashi et al. 2009). As a result, they can maintain good activity at pH levels ranging from 6 to 10 and can lose it at very low pH values (Ghojavand et al. 2008, Gong et al. 2015). However, it can be said that Z5 biosurfactant presents interesting activities at a wide pH range; results similar to those published by (Mnif et al. 2013b) and (Feng et al. 2019).

The study of Z5 biosurfactant activity at different salt concentrations showed an activation of the surface tension reducing capacity with a maximal activation of about 30 % at 5% NaCl (Fig. 6). So we can say that Na^+ cation strongly activates Z5 biosurfactant as observed by (Thimon et al. 1992) for a *B. subtilis* derived surfactin and (Huszcza &Burczyk 2003) for a *B. coagulans* derived biosurfactant. In fact, the molecular surface of the biosurfactant can increase leading to an enhancement of the surface activity (Thimon et al. 1992). However, we observe a gradual inhibition of the surface dispersant activity to loss over of 70 % of its relative activity for 30 % NaCl concentration. Nevertheless, ZNI5 lipopeptide keeps an interesting activity if ever used for the biodispersion of marine water contaminated with hydrocarbons as it remained 60 % of its activity at 3 % NaCl. Similar results were observed for different lipopeptides (Feng et al. 2019, Mnif et al. 2013b, Pathak &Keharia 2014).

Study of biosurfactant stability after pre-incubation at different temperature, pH and salinities

The potential use of biosurfactants in various industrial processes involving thermal treatments requires a high degree of thermal stability to maintain their activities. In this framework, we studied the effect of a one-hour heat treatment at different temperatures ranging from 20 to 100°C on the stability of ZNI5 lipopeptide surface activities. Presented results show that Z5 derived biosurfactant retains about 100 % of its surface tension reducing power and more than 90 % of its oil dispersant activity in the temperature range studied (Fig. 7). Thus, our biosurfactant has a perfect thermostability as observed for different lipopeptides reported in previous bibliographic studies (Chen et al. 2017, Feng et al. 2019, Ghazala et al.

2017, Kiran et al. 2017, Martins et al. 2018, Pathak &Keharia 2014, Purwasena et al. 2019, Zouari et al. 2019).

As presented in Fig. 8, nightly pre-incubations of ZNI5 lipopeptide at different pH levels ranging from 2 to 10 shows that it retains more than 80% of its surface tension reducing power at pH levels from 3 to 10. These results are similar to numerous previous studies reporting the pH stability of the surface activity of bacterial derived lipopeptides (Chen et al. 2017, Feng et al. 2019, Ghazala et al. 2017, Kiran et al. 2017, Martins et al. 2018, Pathak &Keharia 2014, Purwasena et al. 2019, Qiao &Shao 2010, Zouari et al. 2019). However, the oil dispersant activity is greatly attenuated at highly acidic pH values from 2 to 4. It can be explained by the precipitation of the biosurfactant at lower pH values (Martins et al. 2018, Phulpoto et al. 2020, Purwasena et al. 2019, Ravindran et al. 2020, Zhu et al. 2020). These results may be in favor of widening its scope of application in industry and environment.

In order to investigate the potential application of ZNI5 lipopeptide in the bioremediation of oil-contaminated sea waters, it is necessary to study the effect of salinity on its stability. Thus, as observed in Fig. 9, our lipopeptide shows perfect stability after pre-incubations in the presence of high NaCl concentrations confirmed by measurement of their surface tension decrease and oil displacement capacity. Slight decreases in activity are observed at concentrations greater than or equal to 20 %. These results are similar to many previous studies reporting the stability of lipopeptide biosurfactants towards higher NaCl concentration (Chen et al. 2017, Feng et al. 2019, Ghazala et al. 2017, Kiran et al. 2017, Martins et al. 2018, Phulpoto et al. 2020, Purwasena et al. 2019, Ravindran et al. 2020, Zhu et al. 2020, Zouari et al. 2019).

Compared with chemical surfactants, usually inhibited by high NaCl concentration; by thermal treatments and extreme pH values; ZNI5 derived biosurfactant can represents an interesting candidate for the bioremediation of sea waters contaminated by oil spills.

Conclusion

To conclude, a newly lipopeptide biosurfactant producing belonging to *Bacillus* genera was screened and identified by 16S rDNA amplification. The novel strain *Bacillus subtilis* ZNI5 produced three known clusters of lipopeptide belonging to Surfactin, Iturin and Licheniformin isoforms and a newly cluster of isoforms named Inesfactin. The crude lipopeptide preparation had the ability to reduce the surface tension at a minimal value of 32 mN/m with a CMC value of about 350 mg/L. Moreover, it showed a great capacity to disperse motor oil on the surface of water with a maximal displacement diameter of 80 mm at 800 mg/L. Compared with chemical surfactants, our lipopeptide proved better or similar efficiencies suggesting its potential use in bioremediation. The study of the effect of different physic-chemical factors on lipopeptide biosurfactant functional properties proved its great activity and stability. We remarked an interesting thermal and pH activity and stability along with high resistance to different increasing salts concentration. All this properties suggested the potential use of ZNI5 lipopeptide in many fields.

Abbreviations

ZNI5 BioS
Biosurfactant produced by *Bacillus subtilis* ZNI5.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors read the final manuscript and approved its submission to Bioresources and Bioprocessing.

Availability of data and materials

The data sets supporting the conclusions of this article are included in the article.

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Authors' contributions

All authors directly participated in the planning, execution, or analysis of this study. All authors read and approved the final manuscript.

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Competing interest

The authors declare that they have no competing interests.

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Figures

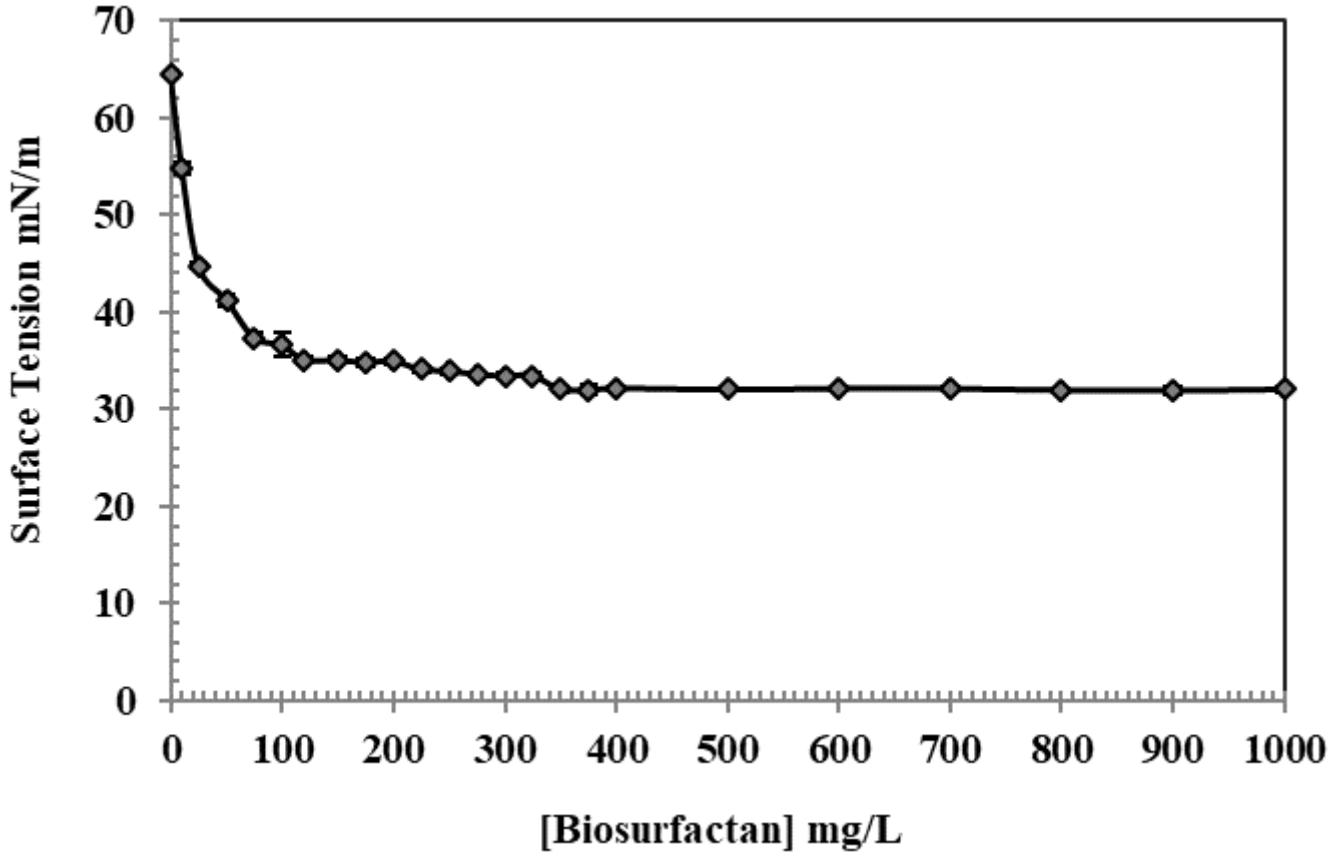

Figure 1

Surface tension decrease versus ZNI5 biosurfactant concentration

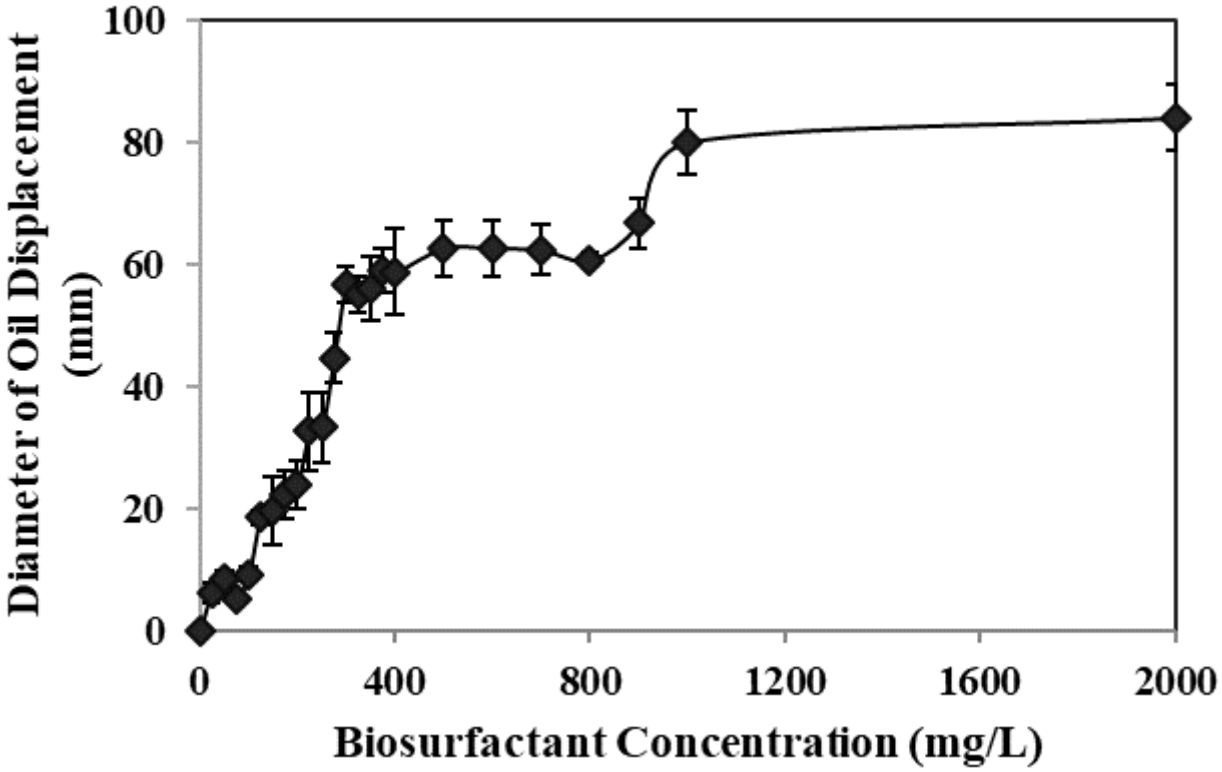

Figure 2

Oil displacement activity increase versus ZNI5 biosurfactant concentration

(a) Motor oil before dispersion

(b) Motor oil after dispersion

Figure 3

Visualization of oil dispersing activity of ZNI5 lipopeptide biosurfactant

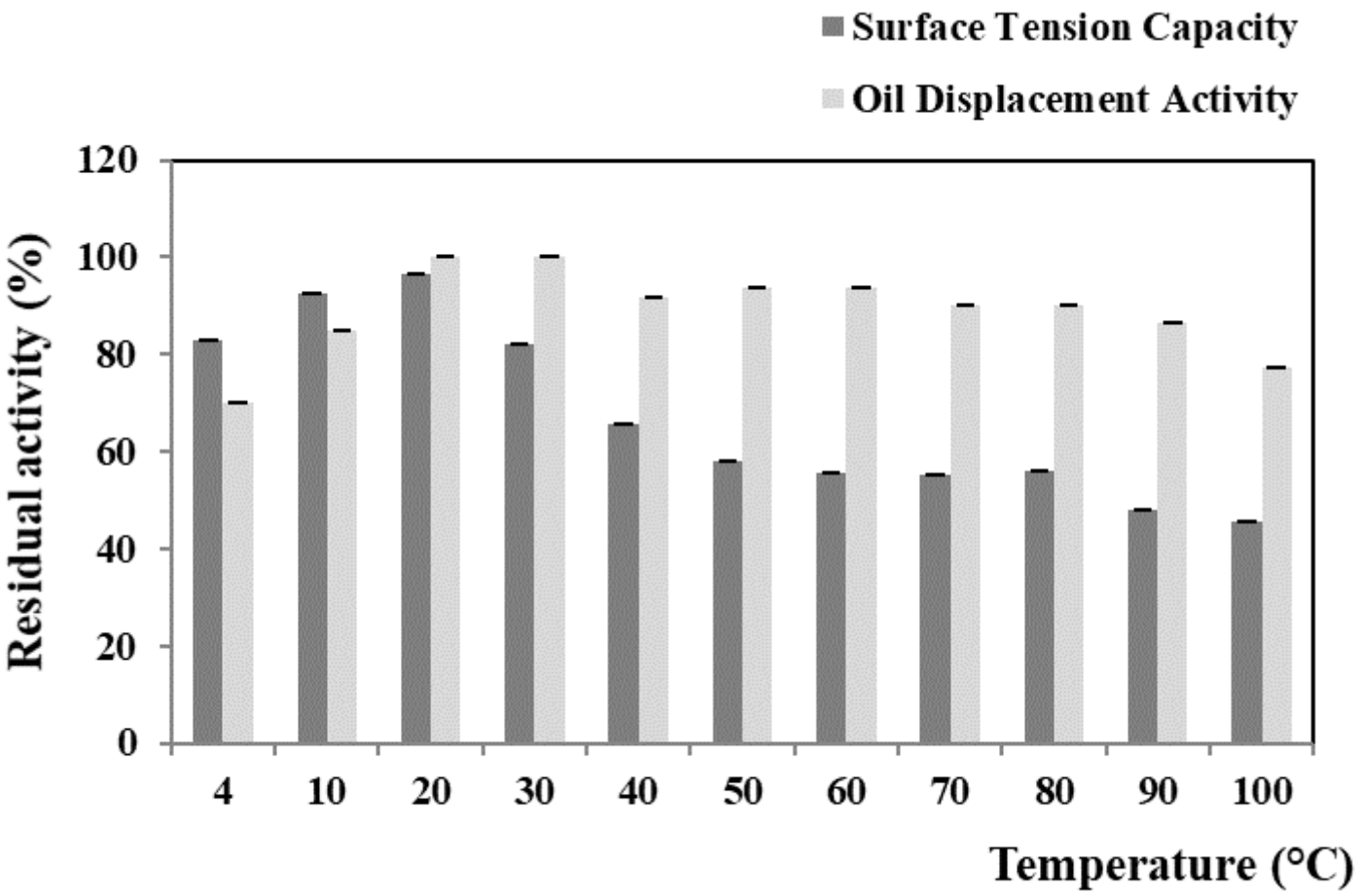

Figure 4

Effect of temperature on ZNI5 lipopeptide biosurfactant activity

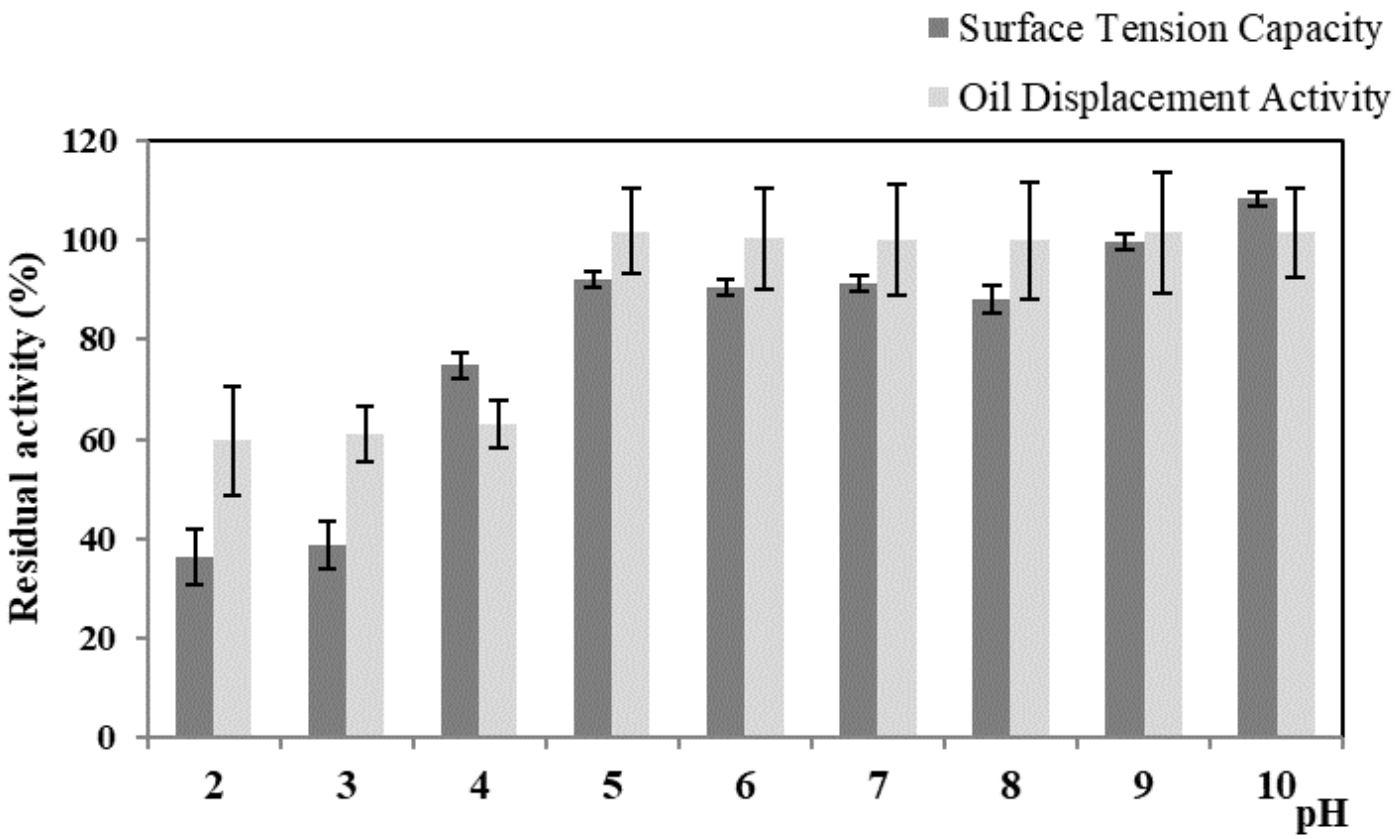

Figure 5

Effect of pH on ZNI5 lipopeptide biosurfactant activity

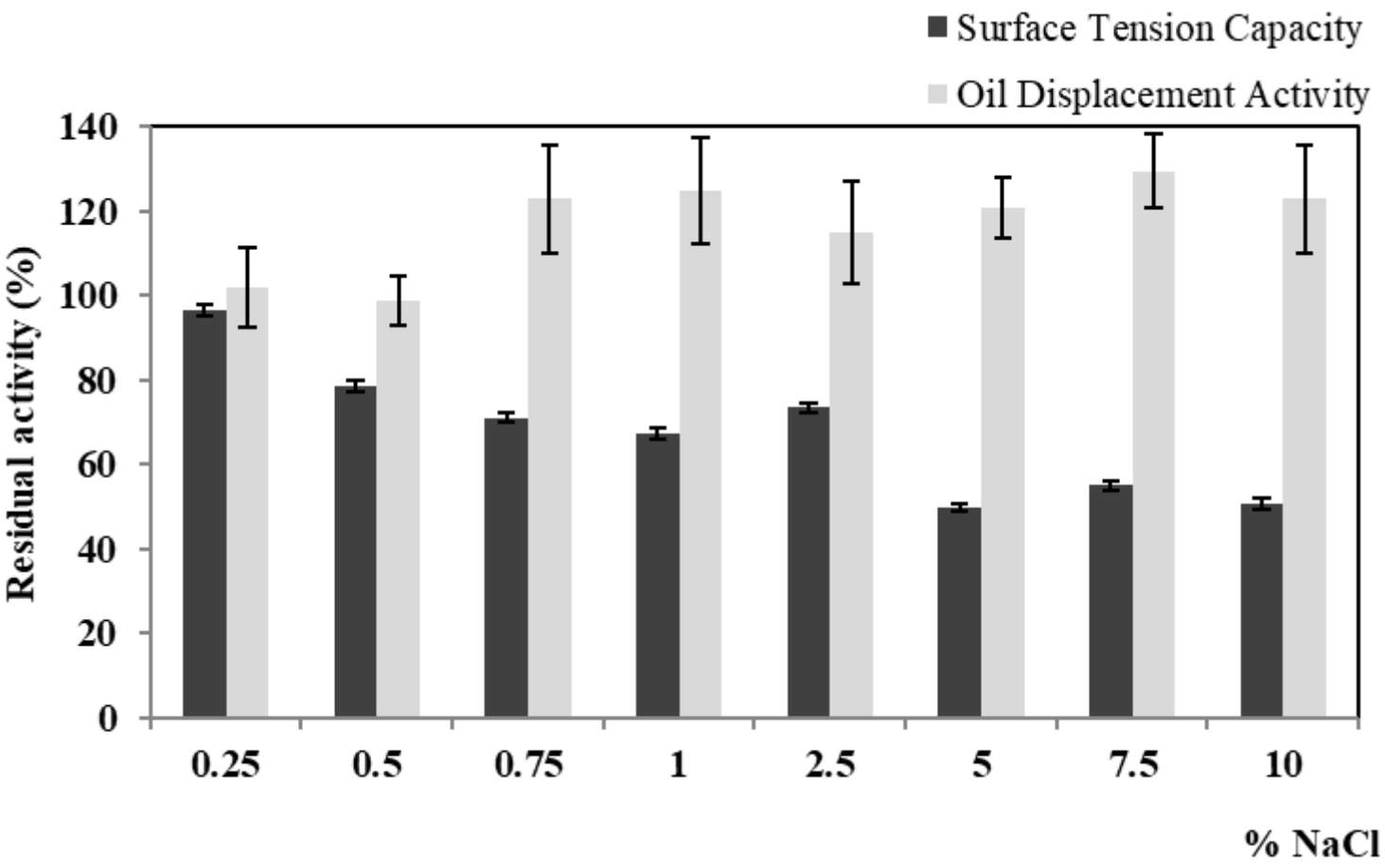

Figure 6

Effect of NaCl ZNI5 lipopeptide biosurfactant activity

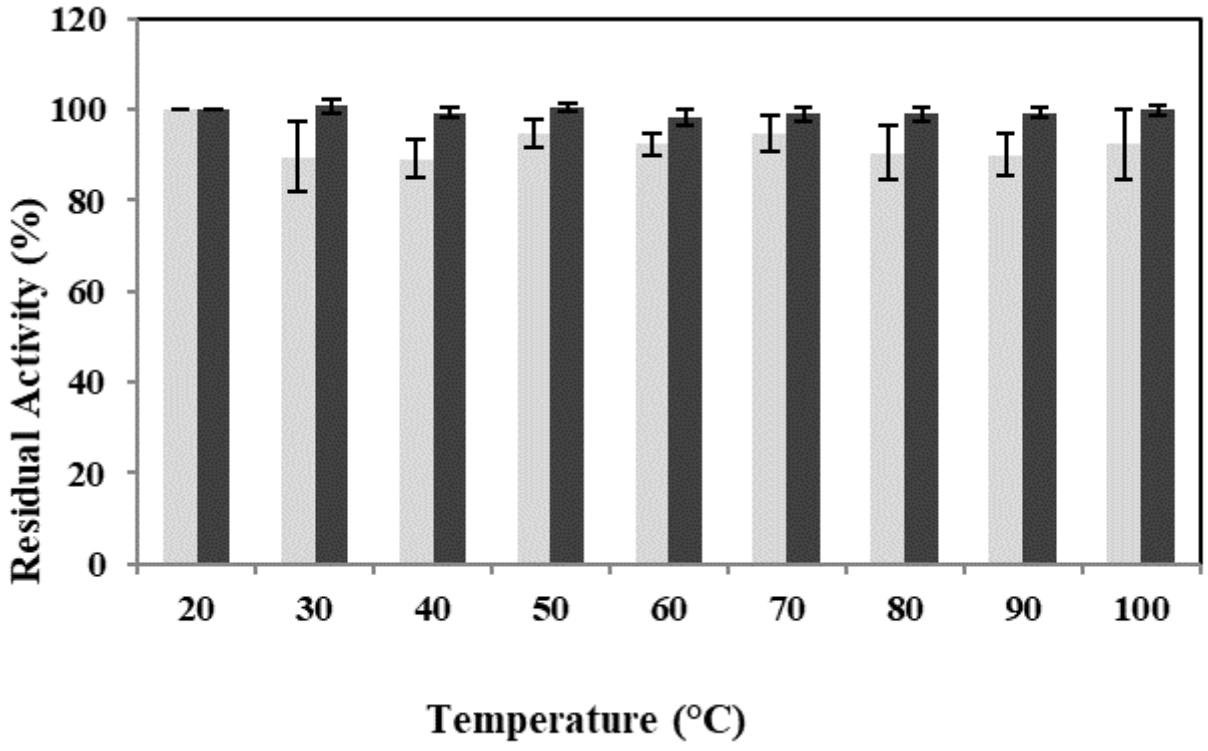

Figure 7

Effect of temperature on ZNI5 lipopeptide biosurfactant stability

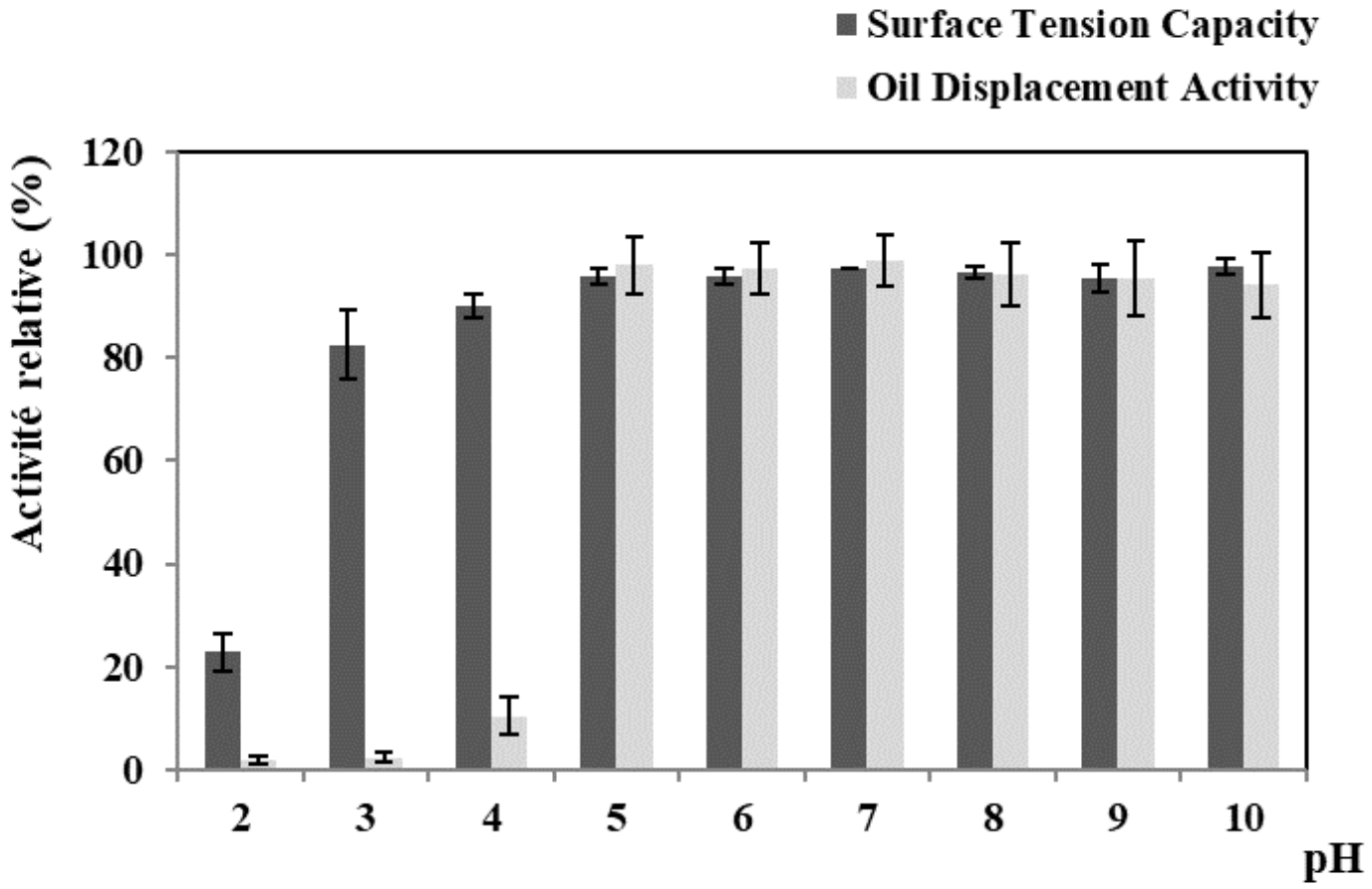

Figure 8

Effect of pH on ZNI5 lipopeptide biosurfactant stability

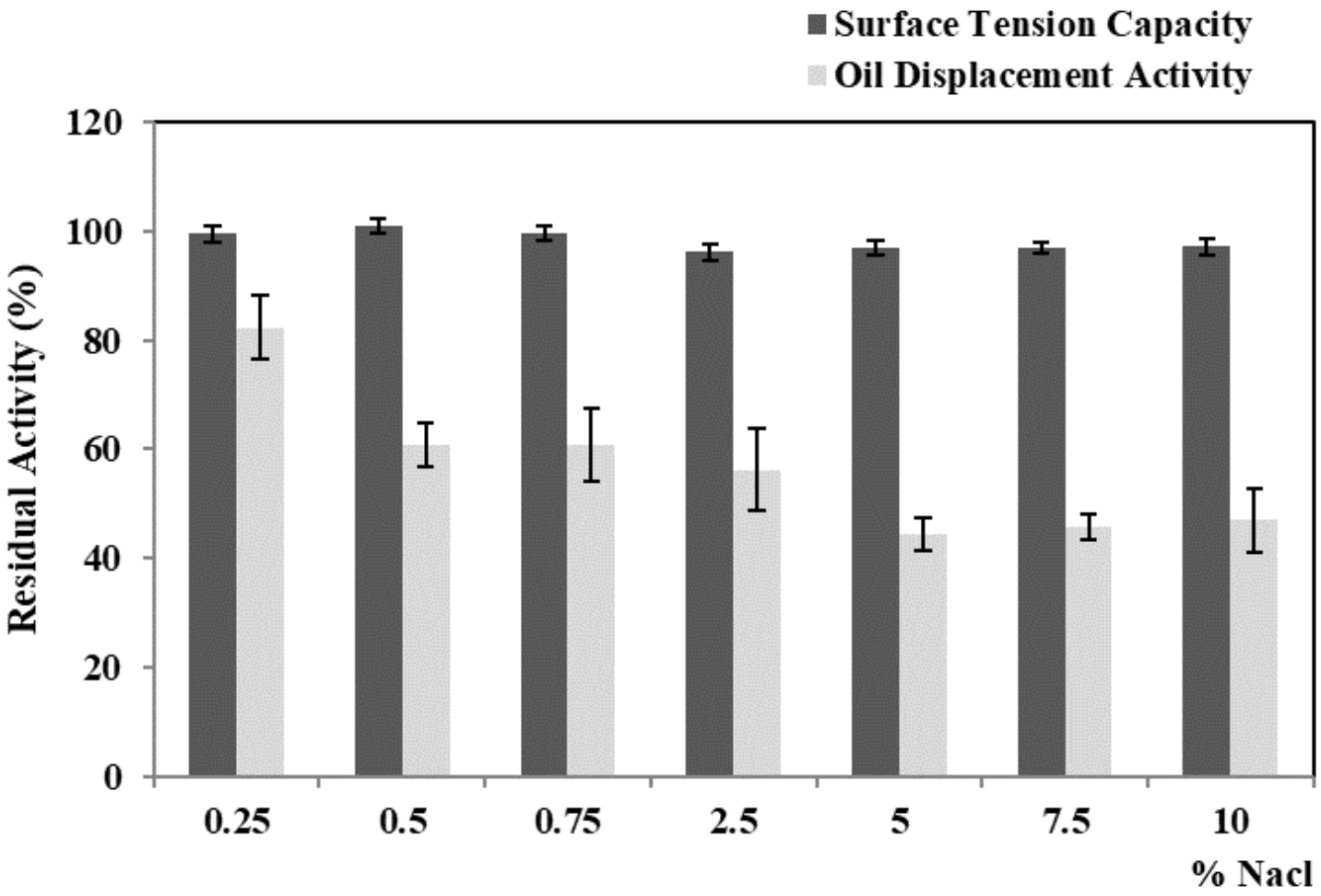

Figure 9

Effect of salinity on ZNI5 lipopeptide biosurfactant stability